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(71) Applicant *(for all designated States except US)*: **PROTEOME SYSTEMS LTD** [AU/AU]; Unit 1, 35-41 Waterloo Road, North Ryde, NSW 2113 (AU).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **PACKER, Nicolle, Hannah** [AU/AU]; 9 Yarabah Avenue, Gordon, NSW 2072 (AU). **KARLSSON, Niclas** [SE/AU]; 5 Fisher Avenue, Ryde, NSW 2112 (AU).

(74) Agent: **F B RICE & CO**; 605 Darling Street, Balmain NSW 2041 (AU).

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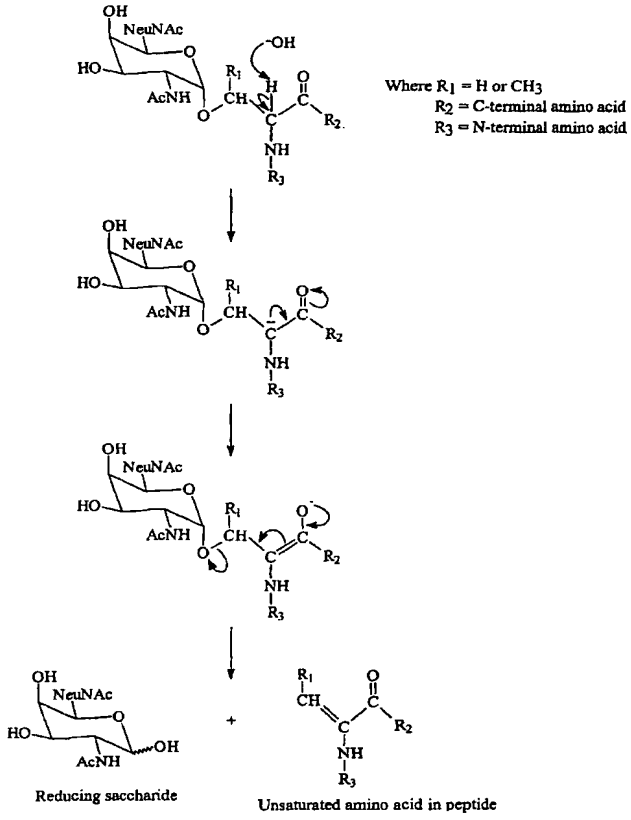
(54) Title: THE RECOVERY OF OXYGEN LINKED OLIGOSACCHARIDES FROM MAMMAL GLYCOPROTEINS



(57) Abstract: The present invention provides a method of recovering O-linked oligosaccharides from a macromolecule, the method comprising the steps: exposing the macromolecule to an alkaline agent to release O-linked olisaccharides from the macromolecule; separating the released oligosaccharide from the macromolecule; and recovering the oligosaccharide.

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The Recovery of Oxygen linked Oligosaccharides from Mammal Glycoproteins.

Technical Field

The present invention relates to methods and systems for removing sugars from macromolecules, particularly the release of oligosaccharides from glycoproteins.

Background Art

Oligosaccharides on glycoproteins are usually found either linked to the hydroxyl group of serine or threonine (O-linked) or asparagine (N-linked). Similarly the glycans (polysaccharides) attached to proteoglycans are also often linked via the hydroxyl group on serine at threonine. So far, the method of choice for releasing of O-linked oligosaccharides from glycoproteins and mucoproteins has been the chemistry of β-elimination in dilute alkali [Carubelli *et al.*, 1965]. The glycans are eliminated by incubation with dilute alkali, resulting in the release of a reducing glycan and the formation of an unsaturated amino acid (Figure 1a). Reducing sugars, however, are unstable in alkali and undergo further β-elimination, known as peeling [Whistler and BeMiller, 1958; Lloyd *et al.*, 1968], with subsequent rearrangement of the terminal residues to saccharinic acids [Stanek *et al.*, 1963] (Figure 1b). To prevent this, 0.8 - 1 M sodium borohydride is normally added [Carlson, 1968], to convert the reducing *O*-glycans to oligosaccharide alditols (Figure 2a). Oligosaccharide alditols are stable to the action of alkali because they do not contain an aldehyde group.

A significant disadvantage of this method is that the resulting glycan alditols are unsuitable for further chemical derivatisation, which severely limits the possibilities for improving their detectability by the inclusion of a chromophore or fluorophore, for example. The addition of a radiolabel to the oligosaccharide alditols by using tritium-labelled borohydride is inherently inefficient because of the high molarity of reducing agent required to prevent peeling, and a large amount of $^3H_2$ gas is produced [Amano and Kobata, 1989].

A further disadvantage of reductive β-elimination is that it does not permit *O*- and *N*-linked glycans to be distinguished. Initially, it was believed that the *N*-glycosidic linkage was relatively stable to alkali [Neuberger *et al.*, 1972] and was only hydrolysed using relatively harsh conditions such as 1 M sodium hydroxide and 1 M sodium borohydride at 100°C for 4-6 hours [Lee

and Scocca, 1972]. Rasilo and Renkonen [1981], however, found that mild alkaline sodium borohydride treatment was capable of releasing the *N*-linked glycans in the form of oligosaccharide-alditols. Ogata and Lloyd [1982] showed that *N*-linked glycans are released initially as glycopeptides, which are then mostly (60 percent) hydrolysed to oligosaccharides. It was subsequently shown that the presence of the borohydride was responsible for the release of the *N*-linked glycans, with the majority being recovered as glyco-asparagines [Argade *et al.*, 1989]. Likhosherstov *et al.* [1990], proposed the inclusion of cadmium acetate to inhibit the reductive cleavage of *N*-glycosidic (and peptide) bonds and permit selective release of *O*-glycans. This method has not been widely accepted, possibly because ethylenediamine-tetra-acetic acid (EDTA) must be added to prevent the formation of solid cadmium hydroxide ($Cd(OH)_2$). The resulting cadmium-EDTA complex interferes with the separation and detection of the glycan alditols.

In 1993, Patel *et al.* described a mild hydrazinolysis method for the release and recovery of both *O*- and *N*-linked glycans, and yet milder conditions for the selective release and recovery of the *O*-linked glycans. The release of *N*-linked glycans required heating in anhydrous hydrazine at 95°C or above, while the removal of the *O*-linked glycans can be achieved at 60°C. The difference resides in the mechanisms involved and there can be overlap of the two reactions which results in non-selective release of both types of glycans. *N*-linked glycans are removed by hydrazinolysis of the amide linkages of asparagine, while removal of the *O*-linked species probably involves a β-elimination process, promoted by the basicity of the hydrazine.

A result of using hydrazine is that, as the sugars are released, they are converted to the hydrazones and protected from peeling under the basic conditions. The glycan hydrazones must then be converted back to the reducing glycans by treatment with copper acetate [Patel *et al.*, 1993; Patel and Parekh, 1994], or mild acid [Williams, 1983] for further derivatisation. This method has not been accepted as a routine way of removing the glycans from mucins or other glyco-molecules. The reasons for this have not been well documented, but the stability of the mucin-type glycans, especially the 1-3 linkage against peeling in hydrazine, the insolubility of mucins in anhydrous hydrazine and the toxicity, and flammability of hydrazine may be some of the reasons. Some peeling of mucin-type *O*-linked glycans

consisting of Gal(β1-3)GalNAc, has been observed when immunoglobulin alpha (IgA) from human serum was treated using the conditions optimised for the release of *O*-linked glycans [Mattu *et al.*, 1998].

Another major disadvantage of hydrazinolysis is the loss of information about the types of sialic acids originally present in the glycoprotein, as the acetyl and glycolyl groups attached to these monosaccharide residues are removed by hydrazine. These differences may be very important, as the presence of *N*-glycolylneuraminic acid may be characteristic of mucins associated with cancer [Devine *et al.*, 1991; Devine and McKenzie, 1992; Hanisch *et al.*, 1996].

Non-reductive release of O-linked oligosaccharides as glycan hydrazones using the mildly alkaline 0.2 M triethylamine in 50% aqueous hydrazine has been described by Cooper *et al* (1994). This method has similar limitations as that described for hydrazinolysis and has not proved to be successful for removal of the oligosaccharides from the highly glycosylated mucins. Similarly, the non-reductive release method described by Chai *et al*, (1997) using 70% w/v aqueous ethylamine requires high temperature to remove the oligosaccharide from porcine gastric mucin and results in extensive peeling and low yields relative to reductive alkaline hydrolysis.

The present inventors have now developed a new means of obtaining sugars from macromolecules containing sugars.

Summary of the Invention

Accordingly, in a first aspect the invention provides a method of recovering O-linked oligosaccharides from a macromolecule, the method comprising the following steps:

(i) exposing the macromolecule to an alkaline agent to release O-linked oligosaccharides from the macromolecule;

(ii) separating the released oligosaccharide from the macromolecule;

(iii) recovering the oligosaccharide.

In a second aspect, the invention provides a method of recovering O-linked oligosaccharides from a macromolecule the method comprising the following steps:

(i) binding the macromolecule to a support;

(ii) contacting the solid support from step (i) with a stream of an alkali agent to release O-linked oligosaccharides into the stream of alkali agent;

(iii) neutralising the alkali agent in the stream; and

(iv) recovering the oligosaccharide.

In a third aspect, the invention provides a system for recovering O-linked oligosaccharides from a macromolecule, the system comprising:

(i) a solid support for immobilising a macromolecule;

(ii) means for providing an alkaline agent to the solid support;

(iii) means for removing the alkaline agent from the solid support;

(iv) means for neutralising the alkaline agent subsequent to its removal from the solid support; and

(v) means for collecting the oligosaccharides.

Brief Description of Figures

Figure 1: Mechanism of alkaline β-elimination for the removal of O-linked glycans from glycoproteins.

Figure 2: Schematic of a) chemistry of β-elimination and b) chemistry of "peeling" reaction.

Figure 3: Comparison of chemistry of a) reductive and b) non-reductive β-elimination.

Figure 4: Diagram of process of non-reductive β-elimination using a system according to the present invention.

Figure 5: Electrospray mass spectrum (ES-MS) of a) non-reduced oligosaccharides released from bovine submaxillary mucin by the system shown in Figure 3, compared with b) reduced oligosaccharides released by reductive β-elimination.

Figure 6: ES –MS of a) non-reduced oligosaccharides released from porcine gastric mucin by the system shown in Figure 3, collected and then reduced, compared with b) reduced oligosaccharides released by reductive β-elimination.

Figure 7: Table of masses obtained by ES –MS of non-reduced oligosaccharides released from porcine gastric mucin by the system shown in Figure 3, collected and then reduced compared with the masses of reduced oligosaccharides released by reductive β-elimination.

Figure 8: Time course of elimination of reducing oligosaccharides from a) bovine submaxillary mucin and b) porcine gastric mucin.

Figure 9: ES-MS of non-reduced oligosaccharides from bovine fetuin.

Figure 10: ES-MS of non-reduced oligosaccharides released from porcine gastric mucin by the system shown in Figure 4, collected and then reacted

with hydroxylamine to tag the available reducing end with a functional group enabling positive ES-MS.

Figure 11: Apparatus comprising a system for recovering O-linked oligosaccharides from a macromolecule.

Figure 12: Solid support apparatus for immobilising a macromolecule, and thermal heating block.

Figure 13: Solid support apparatus for immobilising a macromolecule, chromotography column, means for collecting oligosaccharides and thermal heating block.

Figure 14: Chromotography column, and means for collecting oligosaccharides.

Figure 15: Sectional view of chromotography column, and means for collecting oligosaccharides.

Figure 16: Means for collecting oligosaccharides.

Detailed Description of Invention

In a first aspect the invention provides a method of recovering O-linked oligosaccharides from a macromolecule, the method comprising the following steps:

(i) exposing the macromolecule to an alkaline agent to release O-linked oligosaccharides from the macromolecule;

(ii) separating the released oligosaccharide from the macromolecule;

(iii) recovering the oligosaccharide.

Preferably, the macromolecule is bound to a support.

Preferably, the released oligosaccharide is separated from the macromolecule in association with the alkaline agent and the alkaline agent is neutralised.

In one embodiment, the alkaline agent is neutralised by addition of acid or chromatography cation exchange media.

Preferably, the alkali agent is potassium hydroxide, sodium hydroxide or ammonium hydroxide.

Preferably, the concentration of alkali is 0.05 M - 1.0 M. More preferably, the alkali is 0.05 M - 0.5 M sodium hydroxide.

In one embodiment of the invention the macromolecule is exposed to the alkali agent at about 45°C for about 10 hours to about 40 hours, preferably about 16 hours.

In a second aspect, the invention provides a method of recovering O-linked oligosaccharides from a macromolecule the method comprising the following steps:

(i) binding the macromolecule to a support;

(ii) contacting the solid support from step (i) with a stream of an alkali agent to release O-linked oligosaccharides into the stream of alkali agent;

(iii) neutralising the alkali agent in the stream; and

(iv) recovering the oligosaccharide.

Preferably, the support is a chromatographic material or a membrane or other porous hydrophobic material.

More preferably, the support is reverse phase chromatography beads.

In one embodiment step (iii) comprises passing the stream through a medium which neutralises the alkali agent. Preferably, the medium is chromatography cation exchange media.

In an alternate embodiment step (iii) comprises addition of an acid or chromatography cation exchange media to the stream. Preferably, the acid is hydrochloric acid.

In a preferred embodiment of the first and second aspects, the macromolecule is a glycoprotein.

In a third aspect, the invention provides a system for recovering O-linked oligosaccharides from a macromolecule, the system comprising:

(i) a solid support for immobilising a macromolecule;

(ii) means for providing an alkaline agent to the solid support;

(iii) means for removing the alkaline agent from the solid support;

(iv) means for neutralising the alkaline agent subsequent to its removal from the solid support; and

(d) means for collecting the oligosaccharides.

Preferably, the solid support is a column comprising reversed phase chromatography material capable of binding macromolecules.

Preferably, the means for providing the alkaline agent is a pump and the alkaline agent is an alkaline solution.

In one embodiment the means for neutralising the alkaline agent is a column packed with cation-exchange chromatography material.

In a second embodiment the means for neutralising the alkaline agent is an intersecting flow (stream) of acid.

Preferably, the means for collecting oligosaccharides is a column packed with graphitised carbon.

In one embodiment the carbon is porous graphitised carbon.

In a preferred embodiment the columns are placed in-line.

In a further preferred embodiment the columns are placed in-line and the column packed with porous graphitised carbon is connected to a mass spectrophotometer.

The present invention is particularly useful to obtain from glycoproteins O-linked oligosaccharides which have their reducing terminal monosaccharide still in its reducing configuration. This allows for further derivatisation of the reducing end of the oligosaccharide, thus enabling methods for increasing the detectability by spectroscopic methods either by the addition to the oligosaccharide of either a chromophore, fluorophase, or mass spectrometric ionisable tag.

The analysis of O-linked oligosaccharides attached to glycoproteins has been hampered by both the lack of an enzyme able to universally remove all O-linked oligosaccharides as well as by the lack of sensitivity of the analytical tools available for their analysis. Carbohydrates have little absorbance or fluorescence in either visible or ultra-violet light so the standard spectroscopic procedures are unable to be used. Similarly the use of mass spectrometric analysis is limited by the lack of readily ionisable groups contained in the oligosaccharides and the consequent low sensitivity of detection. Traditionally, in the analysis of glycans, the sensitivity of detection is increased by the covalent attachment to the oligosaccharide of a tag whose properties enhance the particular technique being used. The most reactive functional group on a glycan is the reducing terminus of the sugar. Labelling only this terminal moiety in the oligosaccharide does not alter its

native structure and has the additional benefit of creating a tagged end of the structure which can be located easily.

Alkaline β-elimination is accepted as the most quantitative method for releasing the O-linked oligosaccharides from serine and threonine, but the active reducing terminus is peeled in alkali resulting in the degradation of the glycan structure. Traditionally, the best method for protecting the reducing terminus from this degradation is to form the reduced sugar which is stable in alkali. The reduced terminal monosaccharide however is no longer reactive and cannot be tagged with a group to increase the sensitivity of detection of the oligosaccharide.

The particular value of a preferred system used for the methods of the present invention and illustrated schematically in Figure 4 is in the production of released O-linked oligosaccharides in their reducing form which are able to be further reacted to increase the sensitivity of analysis of glycans. This process can be applied to all O-linked glycoproteins and is demonstrated to be successful even with the highly glycosylated mucin glycoproteins which are known to be difficult to analyse.

In order that the present invention may be more clearly understood preferred forms will be described with reference to the following figures.

As depicted in Figure 4, a system for removing sugars from a macromolecule comprises a solid support 20 for immobilising a macromolecule, a means 5 for providing an alkaline agent; a means 30 for removing the alkaline agent from the solid support; a means 40 for neutralising the alkaline agent; and a means 50 for collecting oligosaccharides.

An apparatus 1 for a system for removing sugars from a macromolecule is depicted in more detail in Figures 11, 12 and 13.

The apparatus 1 comprises a reagent container 10 having a closure 11. The closure 11 has an outlet 12 that receives a proximal end of a flexible tube 13. The flexible tube 13 is received at its distal end by an inlet 14 of an injector 15.

The flexible tube 13 serves to provide fluid connection between the container 10 and the injector 15.

The means 5 for providing an alkaline agent further comprises a pump 7 that is housed within the apparatus 1.

A second flexible tube 17 further extends from the injector 15 at an outlet 16 through an orifice 18 and into sealing engagement with solid support 20.

A screw connector 19 is used to sealingly engage an aperture 21 on an upper surface of the solid support 20.

The solid support 20 is spool-shaped. The solid support 20 has the aperture 21 for receiving the alkaline agent and an outlet (not shown) for releasing the alkaline agent.

The solid support is packed with reverse phase beads, such as R2-reversed phase beads or alternatively may contain a membrane.

The solid support 20 is housed in an insulated heating block 25. The insulated heating block 25 can be machined aluminium. The insulated heating block 25 has a recess 26 configured to receive the solid support 20. The insulated heating block 25 further comprises a heating device 27. The heating device 27 can be a thermofilm.

The solid support 20 has an outlet on its lower surface (not shown) which sealingly engages a first end of a screw connector 23. At a second end the screw connector 23 connects to a means 40 for neutralising the alkaline reagent.

A circular insulating pad 24 having a circular orifice to receive the screw connector 23 is positioned between the solid support 20 and the means 40 for neutralising the alkaline reagent.

As depicted in Figure 4, the means 40 for neutralising the alkaline reagent has a first end 41 and a second end 42. The first end 41 is connected by a tube 43 to the solid support 20.

Alternatively and as depicted in Figures 11, 12 and 13, the first end 41 of the means 40 for neutralising the alkaline reagent can be directly engaged with the solid support 20. In this case, the first end 41 has an orifice 45 to receive the screw connector 23.

The means 40 for neutralising the alkaline agent can be a column packed with cation-exchange chromatography material.

As depicted in Figure 4, the second end 42 of the means 40 for neutralising the alkaline reagent is connected by a tube 44 to a means 50 for collecting oligosaccharides.

Alternatively and as depicted in Figures 11, 12, and 13, the means 40 for neutralising is directly engaged with a means 50 for collecting

oligosaccharides. As depicted in Figures 14, 15 and 16, the means 50 for collecting oligosaccharides is detachably engaged with the means 40 for neutralising the alkaline agent by a screw and washer connector 49.

The means 50 for collecting oligosaccharides can be a column or cartridge packed with graphitised carbon. The graphitised carbon can be porous graphitised carbon.

In a most preferred embodiment and as depicted in Figures 11, 12 and 13, the solid support 20 for immobilising a macromolecule, means 40 for neutralising the alkaline agent; and means 50 for collecting oligosaccharides are longitudinally aligned.

As depicted in Figure 16, the means 50 for collecting oligosaccharides can be detached from the means 40 for neutralising the alkaline agent and connected to a tube 51 which provides an alternate fluid connection.

As depicted in Figures 11, 12 and 13 waste product can be collected in a waste container 60.

In another embodiment as depicted in Figure 16, the means 50 for collecting oligosaccharides can be detached from the waste container 60. . The means 50 for collecting oligosaccharides can be connected to a tube 52.

The means 50 for collecting oligosaccharides can be connected with a mass spectrophotometer by the tube 52.

In order that the nature of the present invention may be better understood preferred uses will be described with reference to the following Examples.

Example 1:

Release of oligosaccharides from bovine submaxillary mucin

Mucins consist of highly glycosylated regions of serine and threonine amino acids. The glycosylation of these regions is varied and the structures of these oligosaccharides are usually analysed after their release from the protein.

Reversed phase Poros™ R2 (polystyrene beads coated with divinyl benzene, PE Biosciences) (10 mg) were added to a solution of 1.0 mg of bovine submaxillary mucin (BSM, Sigma) in 1 ml 9:1 H_2O:ACN. The glycoprotein-coated beads were packed into a (A) cartridge and a solution of 0.05 M potassium hydroxide was pumped through at a flow rate of 0.1

ml/min for 16 hrs at 45°C. The eluent from the reversed phase beads was passed immediately through an in-line cation exchange column (AG50W-X8 4.6mm i.d. × 27 cm , 7.6 meq capacity) which was placed in-line with a conditioned (washed with several column volumes of 80% acetonitrile:0.1% TFA, followed by re-equilibration with water) graphitised carbon cartridge (300 mg). The retained sugars recovered by elution with 2 ml of a pH 9.0 ammonium formate buffer (50 mM) with 25 % acetonitrile were analysed by electrospray ionisation time of flight mass spectrometry (ESI-TOF) (Figure 5a).

The masses of the recovered glycans were compared with the masses of the reduced glycans recovered by conventional reductive β-elimination in which the same amount of BSM was incubated in 0.05M potassium hydroxide, 1.0 M sodium borohydride for 16 hrs at 45°C. This sample was also desalted on a graphitised carbon cartridge before analysis by ESI-TOF (Figure 5b). The same oligosaccharide masses (taking into account the addition of 2 Da upon reduction) were obtained by both methods. The glycosylation pattern with respect to the relative intensities of the molecular ions were also preserved between the two methods of release. The oligosaccharides from bovine submaxillary mucin have been described previously, and the dominating oligosaccharides are the NeuAc/NeuGcα2-6GalNAc and GlcNAcβ1-3(NeuAc/NeuGcα2-6)GalNAc. The similar relative amount of recovery of the latter species in the non reduced sample (Figure 5) and the reduced sample demonstrate that the level of peeling is negligible.

Example 2:

Release of oligosaccharides from porcine gastric mucins (PGM)

Porcine gastric mucins are very heterogenous glycosylated with mainly large neutral oligosaccharide species (Karlsson *et al,* 1997) and sulphated species. The present inventors subjected 1.0 mg of porcine gastric mucin (Sigma) to the same treatment as bovine submaxillary mucins. The potassium hydroxide flow was neutralised with a flow of 0.1 ml/min 0.05 M HCl and collected online on a small Hypercarb (porous graphitised carbon) (Shandon, UK) guard column (10 × 4 mm). The oligosaccharides were eluted with the described gradient for LC-MS analysis for bovine submaxillary mucin oligosaccharides and the porcine gastric oligosaccharides were collected. Half of the sample was reduced in 0.05 M potassium hydroxide,

1.0 M sodium borohydride, and analysed with LC-MS (Figure 6a) as described above for bovine submaxillary mucin oligosaccharides. The sample was compared with porcine gastric mucin oligosaccharides released from 1.0 mg of mucin by 0.05 M potassium hydroxide in presence of 1.0 M sodium borohydride.(Figure 6b). The detected oligosaccharide masses are summarised in Table 1 (Figure 7).

Example 3:

Time course for the β-elimination reaction in flow

Porcine gastric mucins and bovine submaxillary mucins (1.0 mg each) were immobilised on R2-beads and each mucin was subjected individually to β-elimination reaction in flow, neutralising the alkali with an in-line H+-exchange column (AG50W-X8 4.6mm i.d. × 27 cm , 7.6 meq capacity). The oligosaccharides were trapped on graphitised carbon cartridges (300 mg) that was changed after 3, 6 and 27 hours. Samples were eluted as described and subjected to LC-MS as described above, detecting ions in negative mode. The response for the mono-isotopic ion for each oligosaccharide composition [M-H]$^-$-ion was recorded and the reaction was considered to be complete after 27 hours (Figures 8a and 8b), thus setting the sum of the recorded responses for each time point and oligosaccharide species to 100% at 27 hours.

Example 4:

Release of oligosaccharides from bovine fetuin

Bovine fetuin has three sites of O-glycosylation and three sites of N-glycosylation. N-linked glycans are usually removed enzymically but there is no suitable enzyme for the release of the O-linked glycans. The O-linked oligosaccharides from fetuin has been described and are dominated by structures containing sialic acid on the C-3 branch of the protein linking GalNAc. Oligosaccharides were recovered by coating fetuin onto R2-beads as described above for Bovine subaxillary mucin with the on-line cation exchange neutralising column and a porous graphitised carbon cartridge (10 x 4mm). The eluate was introduced directly on-line to the mass spectrometer with a flow of 10 µl/min. Negative molecular ions ([M-H]$^-$-ions) were detected (Figure 9) with the composition of the dominating oligosaccharides described from bovine fetuin. The linkage configuration and sequence in the Figure 9 are assigned from the references illustrating that oligosaccharides

with extension on the C-3 of the proximal GalNAc can be recovered in high yields. Figure 9 also illustrates that O-linked oligosaccharides also can be recovered not only from mucins but also from less glycosylated glycoproteins.

Example 5:

Reaction of reducing terminus to enhance sensitivity of detection

Porcine gastric mucin-oligosaccharides were prepared in the process from 1.0 mg of porcine gastric mucin as described for above . One fourth of the sample was derivatised in 450 µl of 67 mM hydroxylamine hydrochloride (Sigma) and 0.87 M sodiumcyanoborohydride at 50°C for 16 h, and 100 µl was subjected to positive LC-MS. Oligosaccharides was eluted from a small Hypercarb guard column (10 × 4 mm), with a gradient from 0-90 % acetonitrile under 5 min with constant 0.2% formic acid throughout the LC-MS run. Figure 10 illustrates that recovered non-reduced oligosaccharides could be derivatised in order to alter the mass spectrometric properties and increase the sensitivity of detection.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in Australia before the priority date of each claim of this application.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

References

Amano J. and Kobata A. (1989) Quantitative conversion of mucin-type sugar chains to radioactive oligosaccharides. *Methods Enzymol.* **179**, 261-270.

Argade S. P., Davies Jr G. D., Van Halbeek H., and Alhadeef J. A. (1989) The effect of alkaline borohydride treatment on N-linked carbohydrates of glycoproteins. *Glycoconj. J.* **6**, 45-56.

Bertolini M. and Pigman W. (1967) Action of alkali on bovine and ovine submaxillary mucins. *J. Biol. Chem.* **242**, 3776-3781.

Carlson D. M. (1966) Oligosaccharides isolated from pig submaxillary mucin. *J. Biol. Chem.* **241**, 2984-2986.

Carubelli R., Bhavanandan V. P., and Gottschalk A. (1965) Studies on glycoproteins. XI. The O-glycosidic linkage of N-acetylgalactosamine to seryl and threonyl residues in ovine submaxillary gland glycoprotein. *Biochim. Biophys. Acta* **101**, 67-82.

Chai, W., Feizi, T., Yuen, C.T. Lawson, MA. (1997) Nonreductive release of O-linked oligosaccharides from mucin glycoproteins for structure/function assignments as neoglycolipids: application in the detection of novel ligands for E-selectin.*Glycobiology* **7**, 861-872.

Cooper C. A., Packer N. H., and Redmond J. W. (1994) The elimination of O-linked glycans from glycoproteins under non-reducing conditions. *Glycoconj. J.* **11**, 163-167.

Devine P. and McKenzie I. (1992) Mucins: structure, function, and associations with malignancy. *BioEssays* **14**, 619-625.

Devine P. L., Clark B. A., Birrell G. W., Layton G. T., Ward B. G., Alewood P. F., and Mckenzie I. F. C. (1991) The breast tumor-associated epitope defined by monoclonal antibody 3E1.2 is an O-linked mucin carbohydrate containing N-glycolylneuraminic acid. *Cancer Research* **51**, 5826-5836.

Hanisch F. G., Stadie T. R., Deutzmann F., and Peter-Katalinic J. (1996) MUC1 glycoforms in breast cancer-cell line T47D as a model for carcinoma-associated alterations of O-glycosylation. *Eur. J. Biochem.* **236**, 318-327.

Karlsson, N.G., Nordman, H., Karlsson, H., Carlstedt, I and Hansson, G.C. (1997) Glycosylation differences between pig gastric mucin populations: a comparative study of the neutral oligosaccharides using mass spectrometry. *Biochem. J.* **326**, 911-917.

Lee Y. C. and Scocca J. R. (1972) A common structural unit in asparagine-oligosaccharides of several glycoproteins from different sources. *J. Biol. Chem.* **247**, 5753-5758.

Likhosherstov L. M., Novikova O. S., Dereviskaya V. A., and Kochetkov N. K. (1990) A selective method for sequential splitting of O- and N-linked glycans from N,O-glycoproteins. *Carbohydr. Res.* **199**, 67-76.

Lloyd K. O. and Savage A. (1991) High performance anion exchange chromatography of reduced oligosaccharides from sialomucins. *Glycoconj. J.* **8**, 493-498.

Mattu T. S., Pleass R. J., Willis A. C., Kilian M., Wormald M. R., Lellouch A. C., Rudd P. M., Woof J. M., and Dwek R. A. (1998) The glycosylation and structure of human serum IgA1, Fab and Fc regions and the role of N-glycosylation on Fc-alpha receptor interactions. *J. Biol. Chem.* **273**, 2260-2272.

Neuberger A., Gottschalk A., Marshall R. D., and Spiro R. G. (1972) Carbohydrate-peptide linkages in glycoproteins and methods for their elucidation, in *Glycoproteins: their composition, structure and function Vol. 2* (Gottschalk A., ed), pp. 450-490. Elsevier, Amsterdam.

Ogata Shun-I. and Lloyd K. O. (1982) Mild alkaline borohydride treatment of glycoproteins - a method for liberating both N- and O-linked carbohydrate chains. *Anal. Biochem.* **119**, 351-359.

Patel T. P. and Parekh R. B. (1994) Release of oligosaccharides from glycoproteins by hydrazinolysis. *Methods Enzymol.* **230**, 57-66.

Patel T., Bruce J., Merry A., Bigge C., Wormald M., Jaques A., and Parekh R. (1993) Use of hydrazine to release in intact and unreduced form both N- and O-linked oligosaccharides from glycoproteins. *Biochemistry* **32**, 679-693.

Rasilo Maija-L. and Renkonen O. (1981) Mild alkaline borohydride treatment liberates N-acetylglucosamine-linked oligosaccharide chains of glycoproteins. *FEBS Lett.* **135**, 38-42.

Stanek J., Cerny M., Kocourek J., and Pacak J. (1963) *The Monosaccharides.* Academic Press; and Publishing House of The Czechoslovak Academy of Sciences, New York/London/Prague.

Whistler R. J. and BeMiller J. N. (1958) Alkaline degradation of polysaccharides. *Adv. Carbohydr. Chem. Biochem.* **13**, 289-329.

Williams J. M. (1983) Tautomerism of saccharide hydrazones in solution and their reaction with nitrous acid. *Carbohydr. Res.* **117**, 89-94.

CLAIMS :-

1. A method of recovering O-linked oligosaccharides from a macromolecule, the method comprising the following steps:

(i) exposing the macromolecule to an alkaline agent to release O-linked oligosaccharides from the macromolecule;

(ii) separating the released oligosaccharide from the macromolecule;

(iii) recovering the oligosaccharide.

2. The method according to claim 1, wherein the macromolecule is bound to a support.

3. The method according to claim 1 or claim 2 wherein the released oligosaccharide is separated from the macromolecule in association with the alkaline agent and the alkaline agent is neutralised.

4. The method according to claim 3 wherein the alkaline agent is neutralised by addition of acid or chromatography cation exchange media.

5. The method according to any one of claims 1 to 4 wherein the alkali agent is potassium hydroxide, sodium hydroxide or ammonium hydroxide.

6. The method according to any one of claims 1 to 5 wherein the concentration of alkali is 0.05 M - 1.0 M.

7. The method according to any one of claims 1 to 6 wherein the alkali is 0.05 M - 0.5 M sodium hydroxide.

8. The method according to any one of claims 1 to 7 wherein the macromolecule is exposed to the alkali agent at about 45°C for about 10 hours to about 40 hours preferably about 16 hours.

9. A method of recovering O-linked oligosaccharides from a macromolecule the method comprising the following steps:

(i) binding the macromolecule to a support;

(ii) contacting the solid support from step (i) with a stream of an alkali agent to release O-linked oligosaccharides into the stream of alkali agent;

(iii) neutralising the alkali agent in the stream; and

(iv) recovering the oligosaccharide.

10. The method according to claim 9 wherein the support is a chromatographic material or a membrane.

11. The method according to claim 9 wherein the support is reverse phase chromatography beads.

12. The method according to any one of claims 9 to 11 wherein step (iii) comprises passing the stream through a medium which neutralises the alkali agent.

13. The method according to claim 12 wherein the medium is chromatography cation exchange media.

14. The method according to any one of claims 9 to 11 wherein step (iii) comprises addition of an acid or chromatography cation exchange media to the stream.

15. The method according to claim 14 wherein the acid is hydrochloric acid.

16. The method according to any one of claims 1 to 15 wherein the macromolecule is a glycoprotein.

17. A system for recovering O-linked oligosaccharides from a macromolecule, the system comprising:

(i) a solid support for immobilising a macromolecule;

(ii) means for providing an alkaline agent to the solid support;

(iii) means for removing the alkaline agent from the solid support;

(iv) means for neutralising the alkaline agent subsequent to its removal from the solid support; and

(d) means for collecting the oligosaccharides.

18. The system according to claim 17 wherein the solid support is a column comprising reversed phase chromatography material capable of binding macromolecules.

19. The system according to claim 17 or 18 wherein the means for providing the alkaline agent is a pump and the alkaline agent is an alkaline solution.

20. The system according to any one of claims 17 to 19 wherein the means for neutralising the alkaline agent is a column packed with cation-exchange chromatography material.

21. The system according to any one of claims 17 to 20 wherein the means for collecting oligosaccharides is a column packed with graphitised carbon.

22. The system according to any one of claims 17 to 21 wherein the columns are placed in-line.

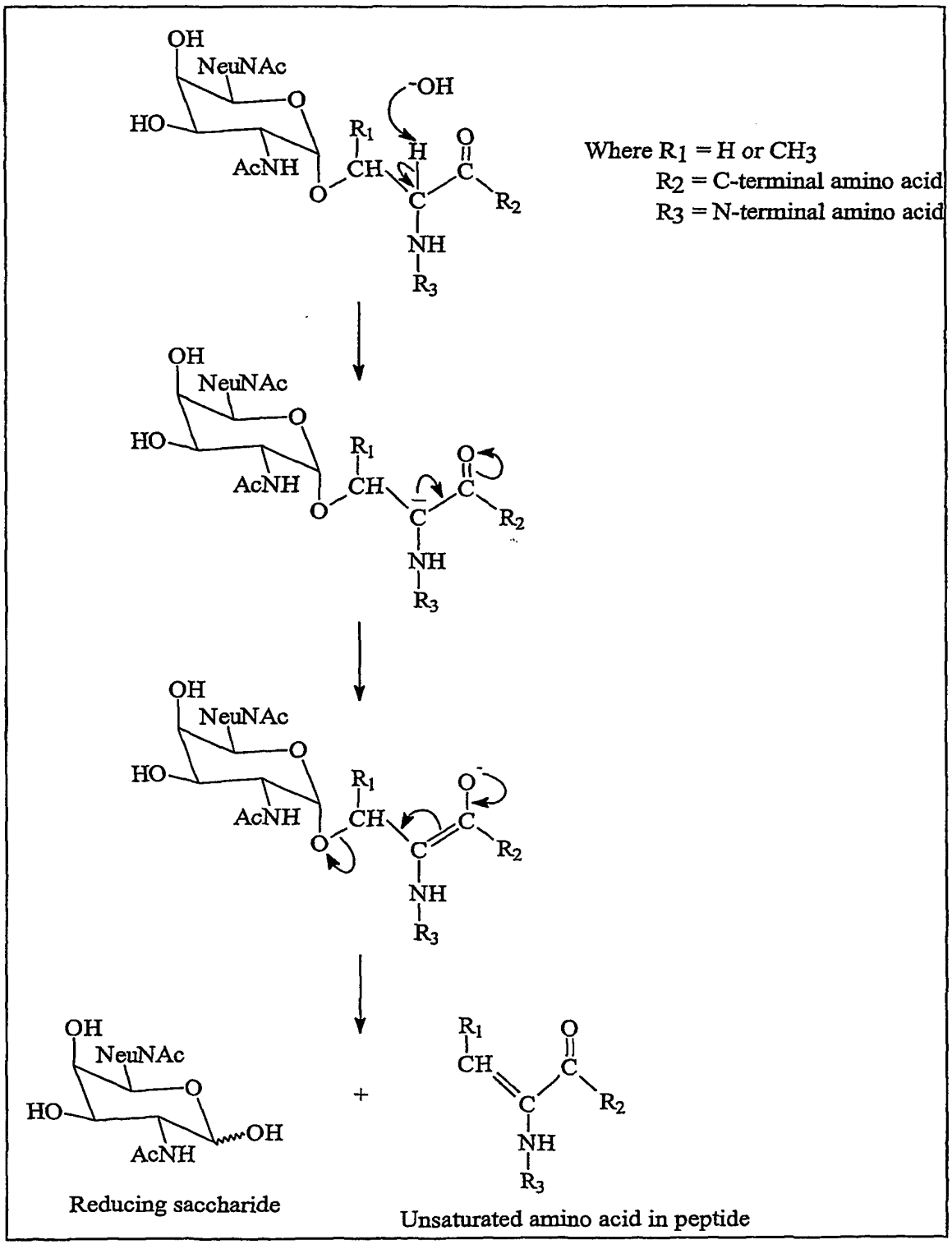
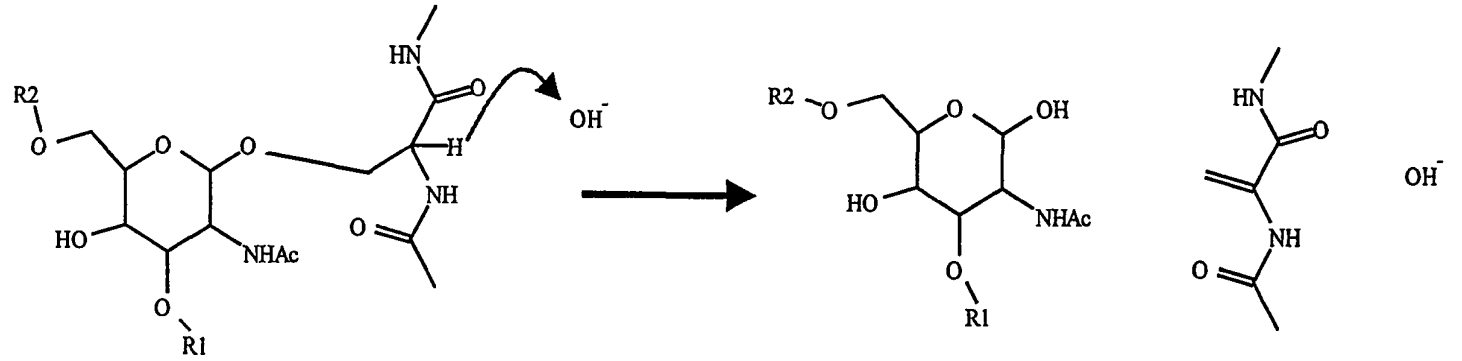

OH
NeuNAc
HO
AcNH
-OH
R1
H
CH
O
C
R2
NH
R3
Where R1 = H or CH3
R2 = C-terminal amino acid
R3 = N-terminal amino acid
Reducing saccharide
+
Unsaturated amino acid in peptide

Fig.1

β-elimination and "Peeling"

a)

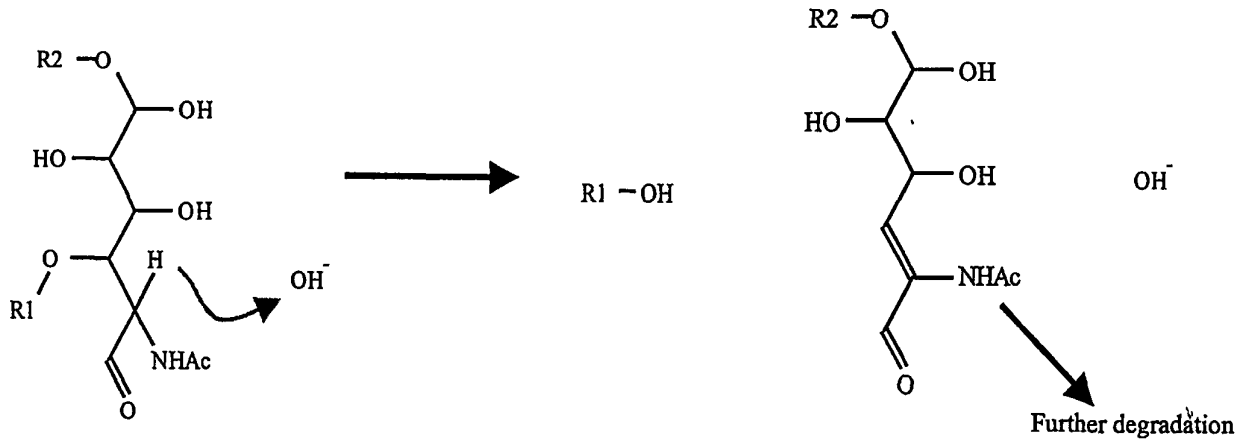
1) β-elimination



O-linked Oligosaccharide Protein Backbone

b)

2) "Peeling"

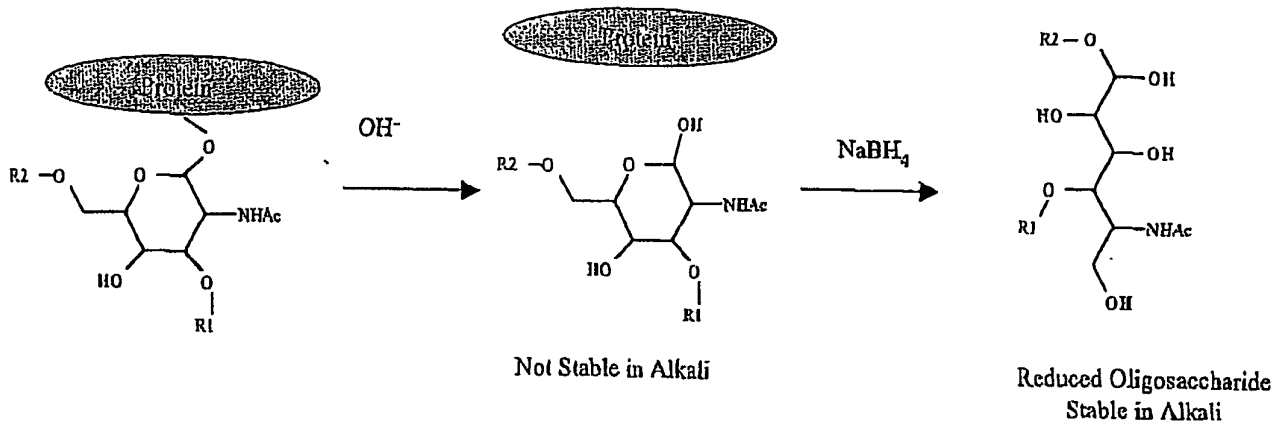


O-linked Oligosaccharide (linear form) C3-branch of Oligosaccharide Degraded Oligosaccharide

Fig.2

Reductive β-elimination versus Non Reductive β-elimination in flow

(a) Reductive β-elimination in solution



(b) Non Reductive β-elimination in flow

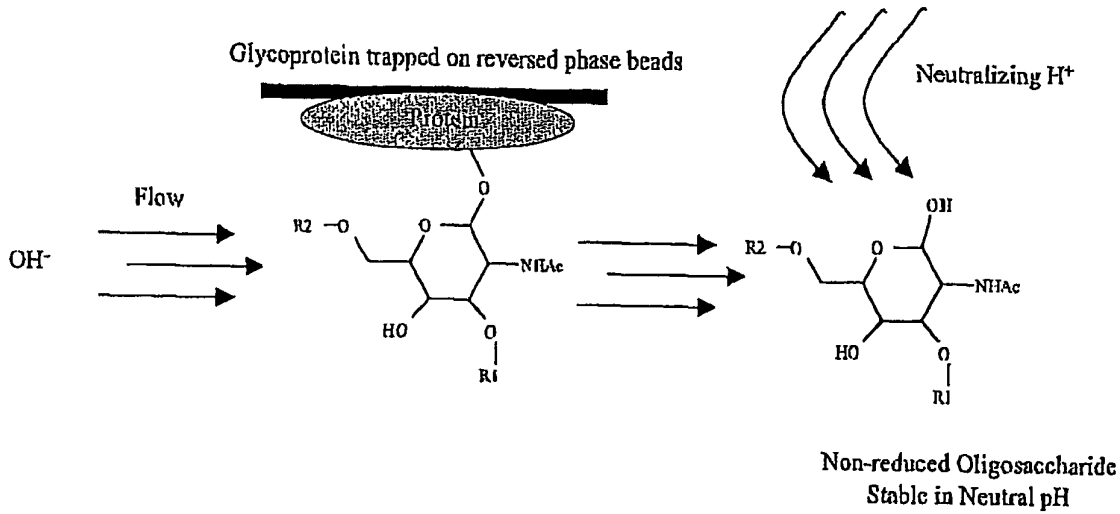


Fig3

Release of O-linked Oligosaccharides

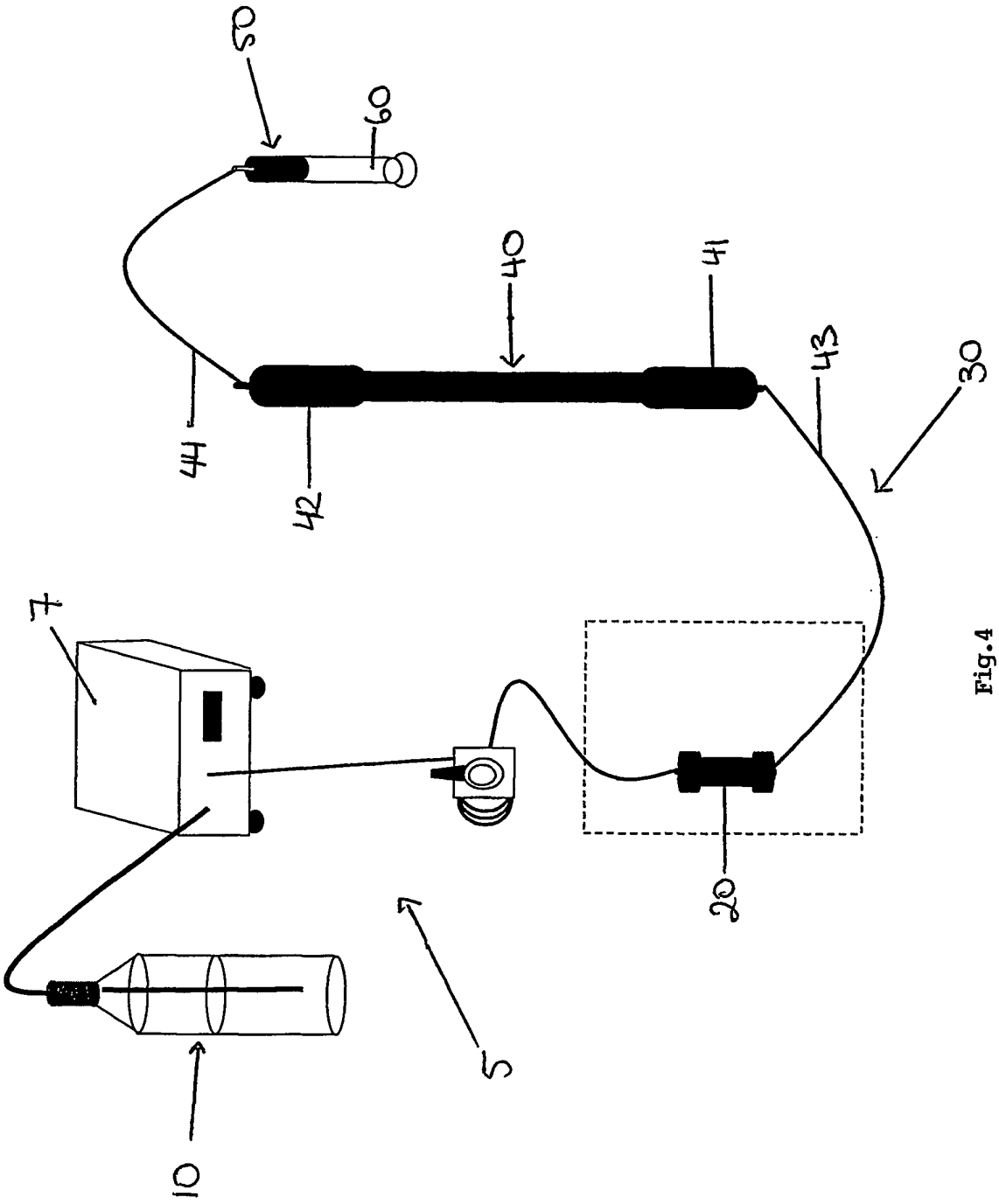
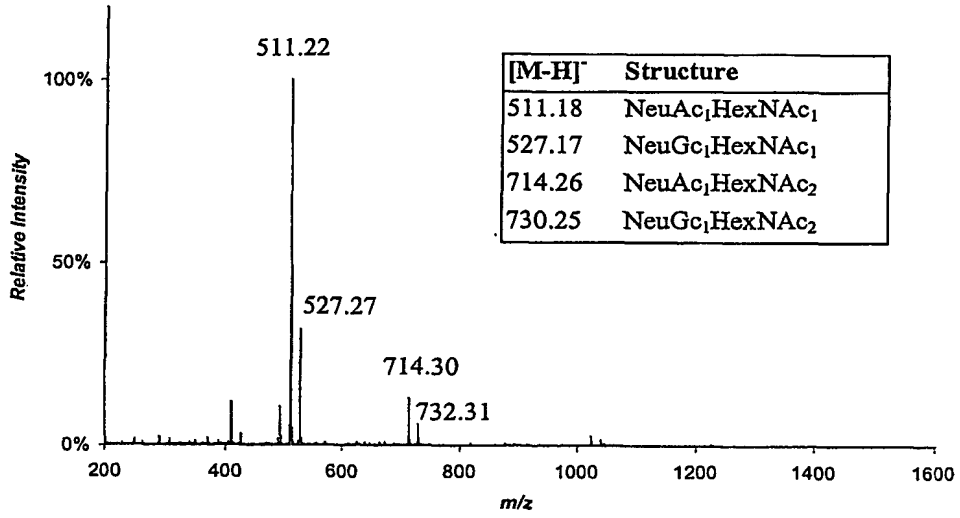


Fig.4

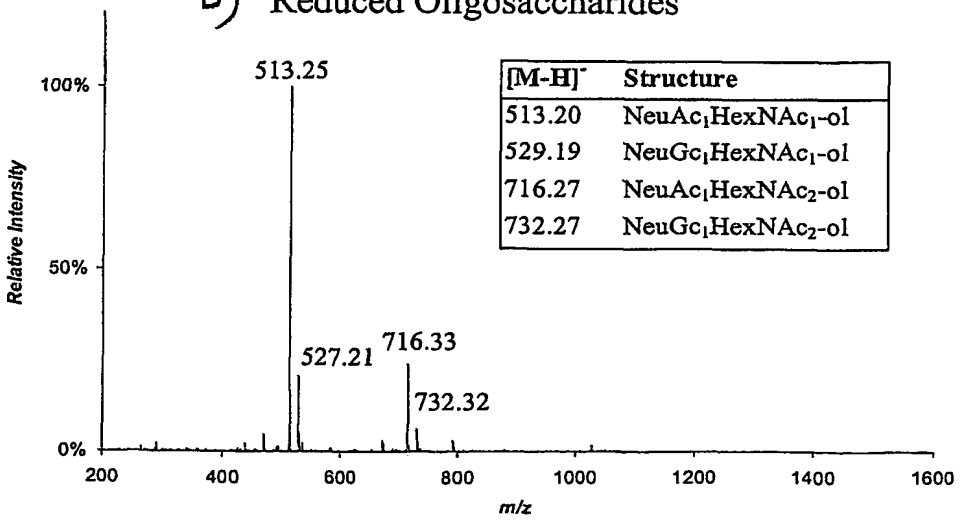
Negative ES-MS of Bovine Submaxillary Mucin

a) Non-reduced Oligosaccharides



[M-H]⁻	Structure
511.18	$NeuAc_1HexNAc_1$
527.17	$NeuGc_1HexNAc_1$
714.26	$NeuAc_1HexNAc_2$
730.25	$NeuGc_1HexNAc_2$

b) Reduced Oligosaccharides

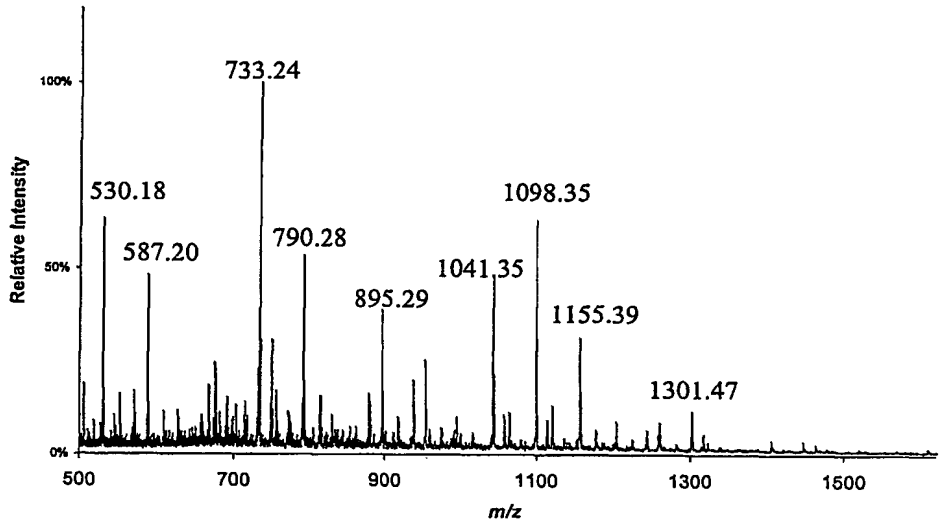


[M-H]⁻	Structure
513.20	$NeuAc_1HexNAc_1$-ol
529.19	$NeuGc_1HexNAc_1$-ol
716.27	$NeuAc_1HexNAc_2$-ol
732.27	$NeuGc_1HexNAc_2$-ol

Fig.5

Negative ES-MS of Porcine Gastric Mucin Oligosaccharides

a) Non-Reduced Oligosaccharides after Reduction



b) Reduced Oligosaccharides

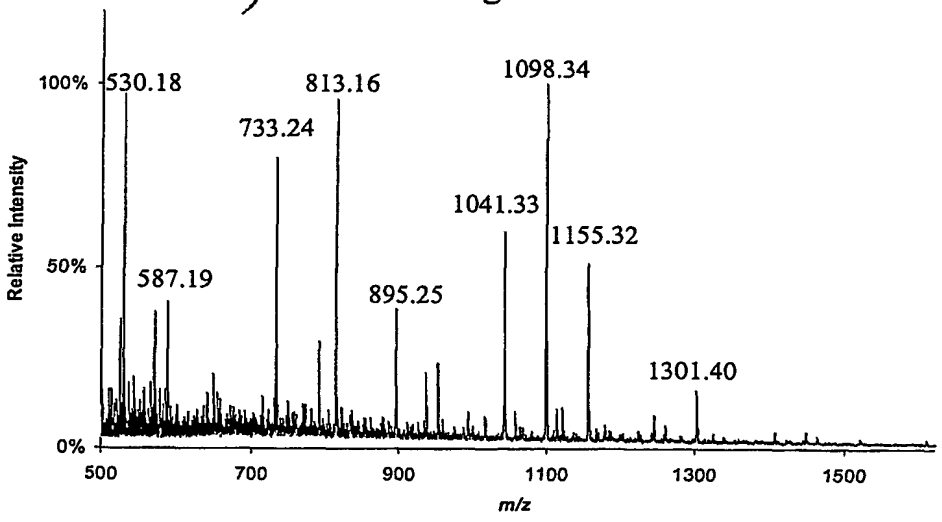


Fig. 6

Detected Porcine Gastric Mucin Oligosaccharides by Negative ES-MS

Calculated [M-H]⁻-ion	Composition	Reductive β-elimination		β-elimination in flow and reduction	
		Charge	Δ(amu)	Charge	Δ(amu)
530.21	$Fuc_1Hex_1HexNAc_1$-ol	1	-0.028	1	-0.028
587.23	$Hex_1HexNAc_2$-ol	1	-0.080	1	-0.04
733.29	$Fuc_1Hex_1HexNAc_2$-ol	1	-0.048	1	-0.048
749.28	$Hex_2HexNAc_2$-ol	1	-0.052	1	-0.012
790.31	$Hex_1HexNAc_3$-ol	1	-0.079	1	-0.029
813.24	HSO_3-$Fuc_1Hex_1HexNAc_2$-ol	1	-0.084	1	-0.084
895.34	$Fuc_1Hex_2HexNAc_2$-ol	1	-0.090	1	-0.05
936.37	$Fuc_1Hex_1HexNAc_3$-ol	1	-0.117	1	-0.037
952.36	$Hex_2HexNAc_3$-ol	1	-0.092	1	-0.062
993.39	$Hex_1HexNAc_4$-ol	1	-0.018	1	-0.098
1041.40	$Fuc_2Hex_2HexNAc_2$-ol	1	-0.068	1	-0.048
1098.42	$Fuc_1Hex_2HexNAc_3$-ol	1	-0.080	1	-0.07
1121.36	HSO_3-$Fuc_2Hex_2HexNAc_2$-ol	1	-0.085	1	-0.065
1155.44	$Hex_2HexNAc_4$-ol	1	-0.121	1	-0.051
1155.44	$Hex_2HexNAc_4$-ol	2	-0.073		
1178.38	HSO_3-$Fuc_1Hex_2HexNAc_3$-ol	1	-0.057	1	-0.057
1244.48	$Fuc_2Hex_2HexNAc_3$-ol	1	-0.088	1	-0.078
1301.50	$Fuc_1Hex_2HexNAc_4$-ol	1	-0.099	1	-0.029
1301.50	$Fuc_1Hex_2HexNAc_4$-ol	2	-0.071		
1406.53	$Fuc_2Hex_3HexNAc_3$-ol	1	-0.080	1	-0.08
1447.56	$Fuc_2Hex_2HexNAc_4$-ol	1	-0.117	1	-0.047
1447.56	$Fuc_2Hex_2HexNAc_4$-ol	2	-0.109		
1463.55	$Fuc_1Hex_3HexNAc_4$-ol	1	-0.184	1	-0.012
1463.55	$Fuc_1Hex_3HexNAc_4$-ol	2	-0.082		
1520.57	$Hex_3HexNAc_5$-ol	1	-0.203	1	-0.073
1609.61	$Fuc_2Hex_3HexNAc_4$-ol	1	-0.190	1	-0.07
1609.61	$Fuc_2Hex_3HexNAc_4$-ol	2	-0.122		
1723.65	$Hex_3HexNAc_6$-ol	2	-0.145	2	-0.065

Fig. 7

Time course for the release of O-linked nonreducing Oligosaccharides

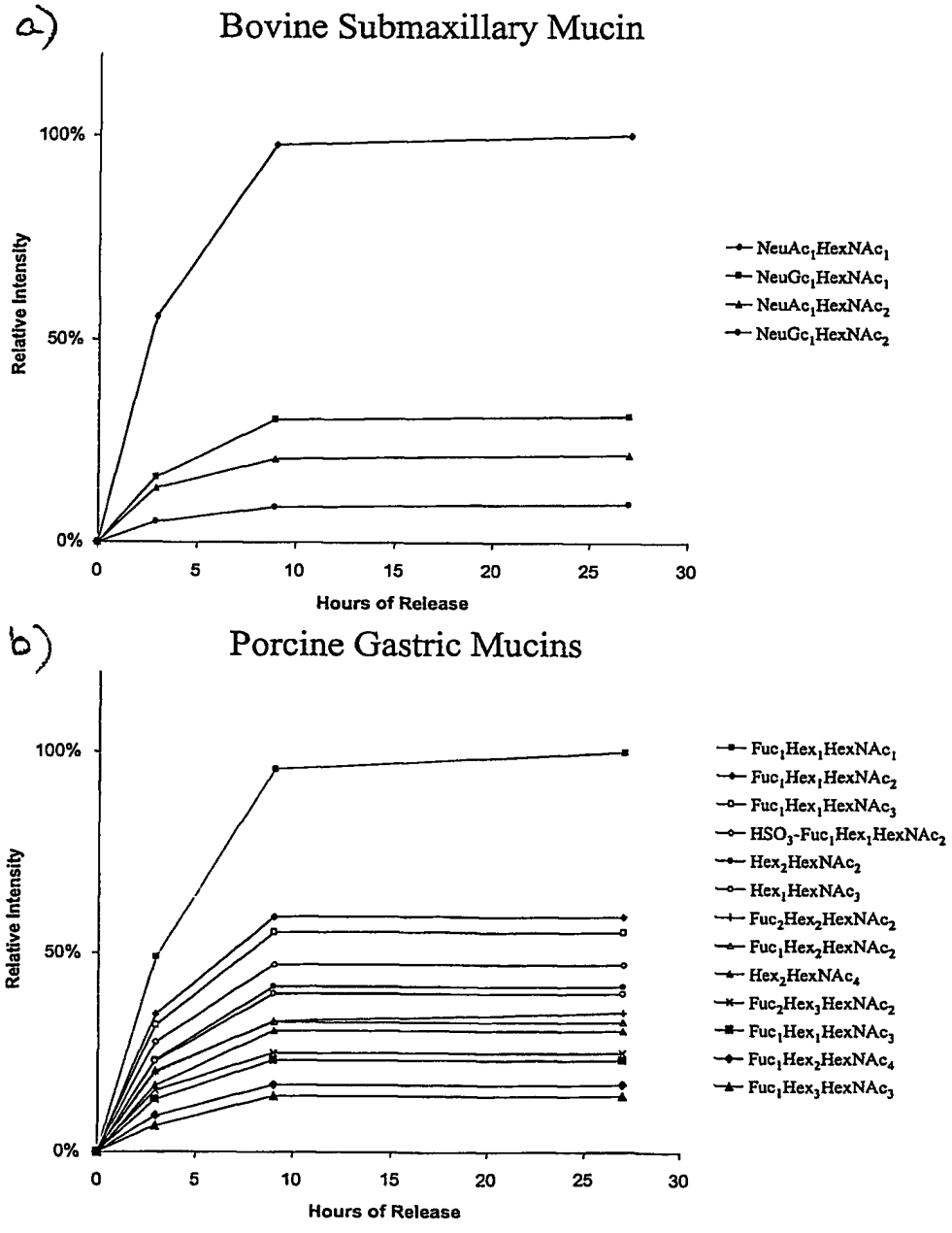


Fig.8

Negative ES-MS of *O*-linked Fetuin Oligosaccharides

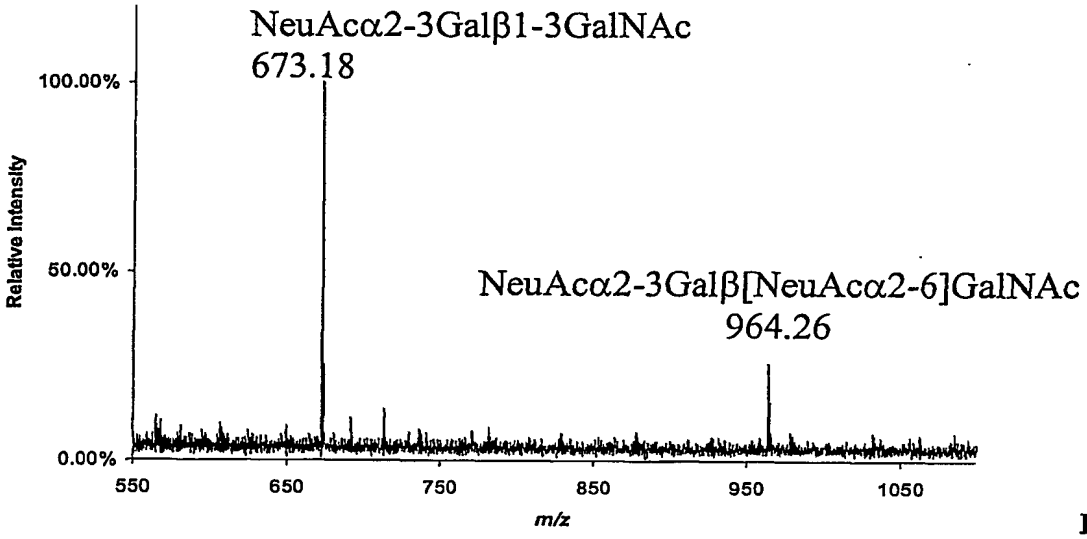


Figure 9

Positive ES-MS of hydroxylamine labelled PGM-oligosaccharides $[M+H]^+$-ions

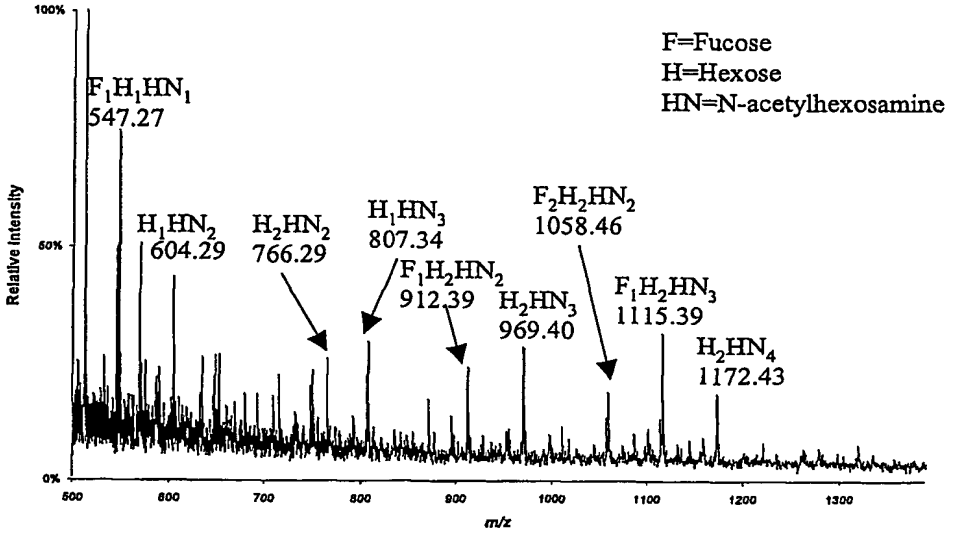


Fig.10

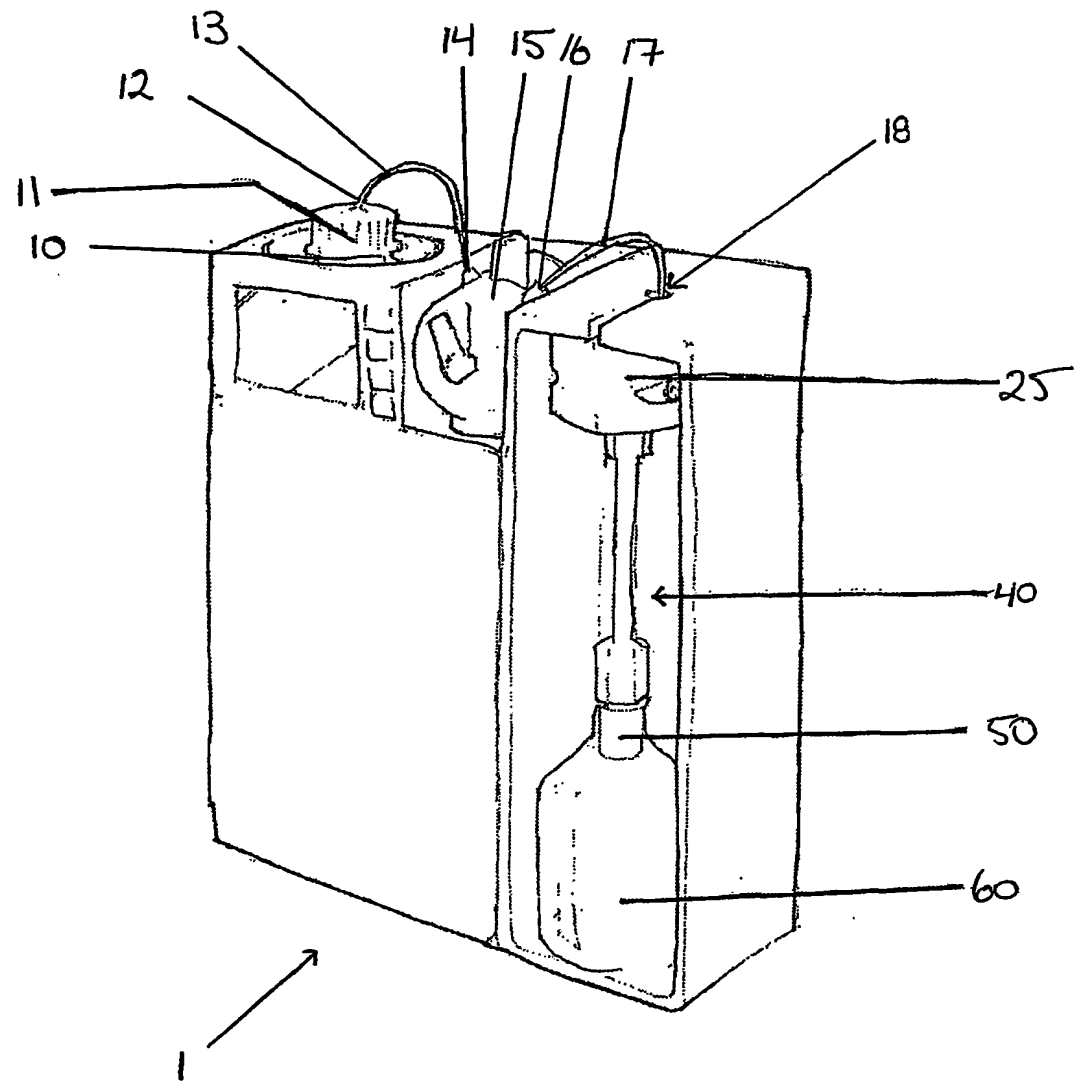


Fig.11

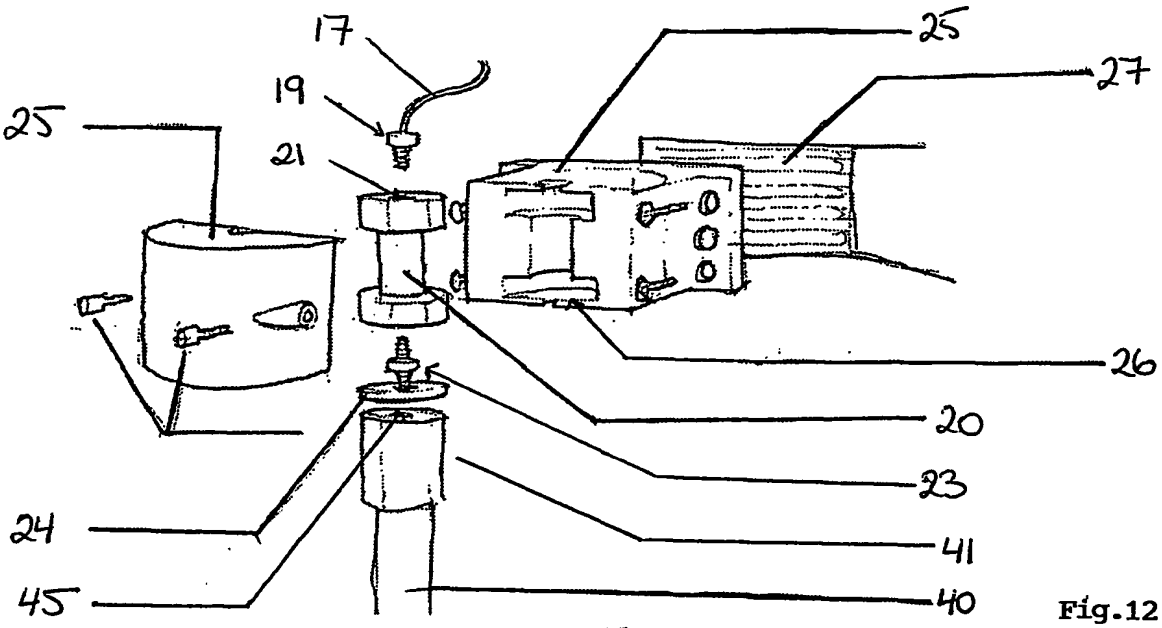


Fig.12

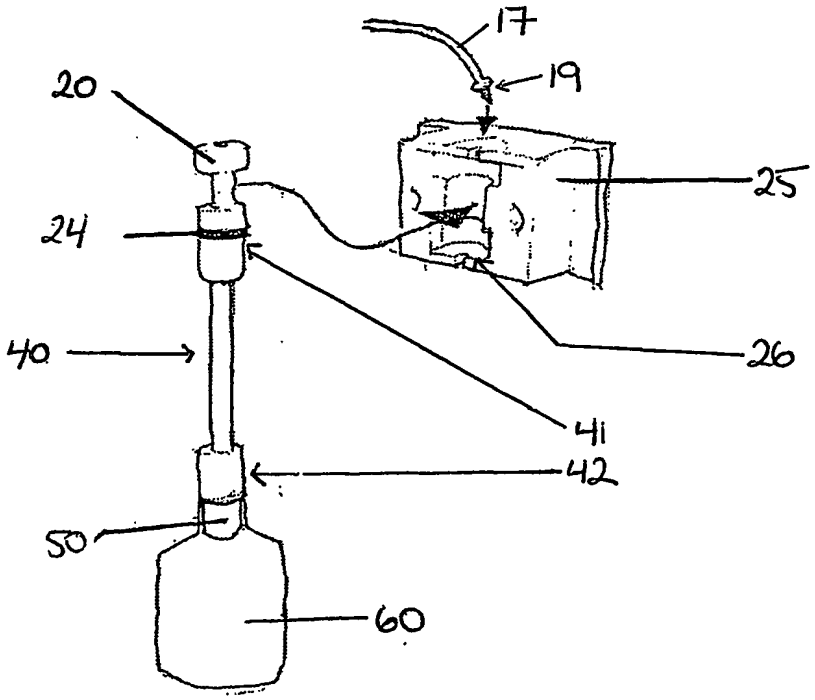


Fig.13

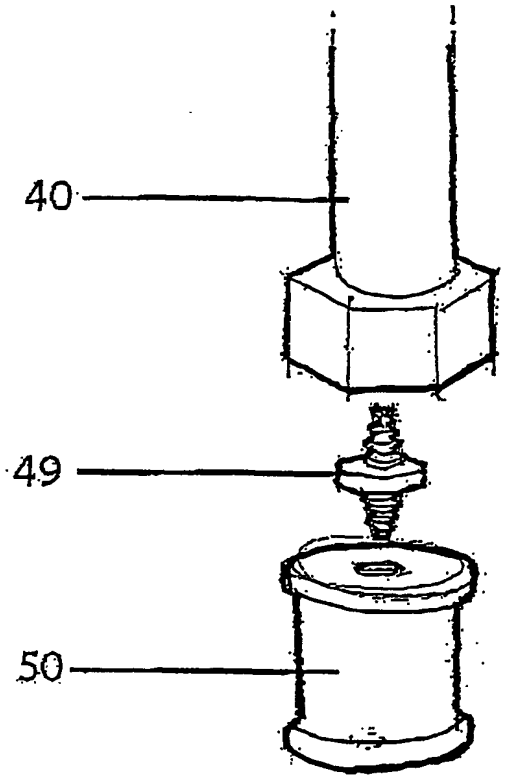


Fig.14

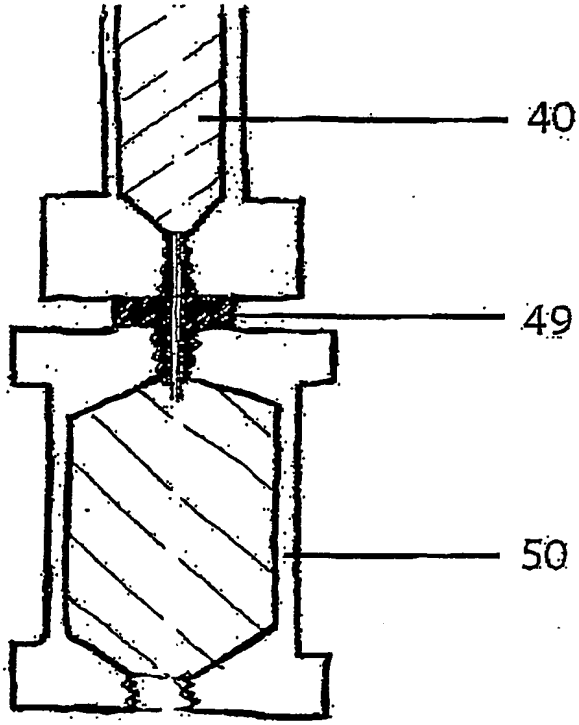


Fig.15

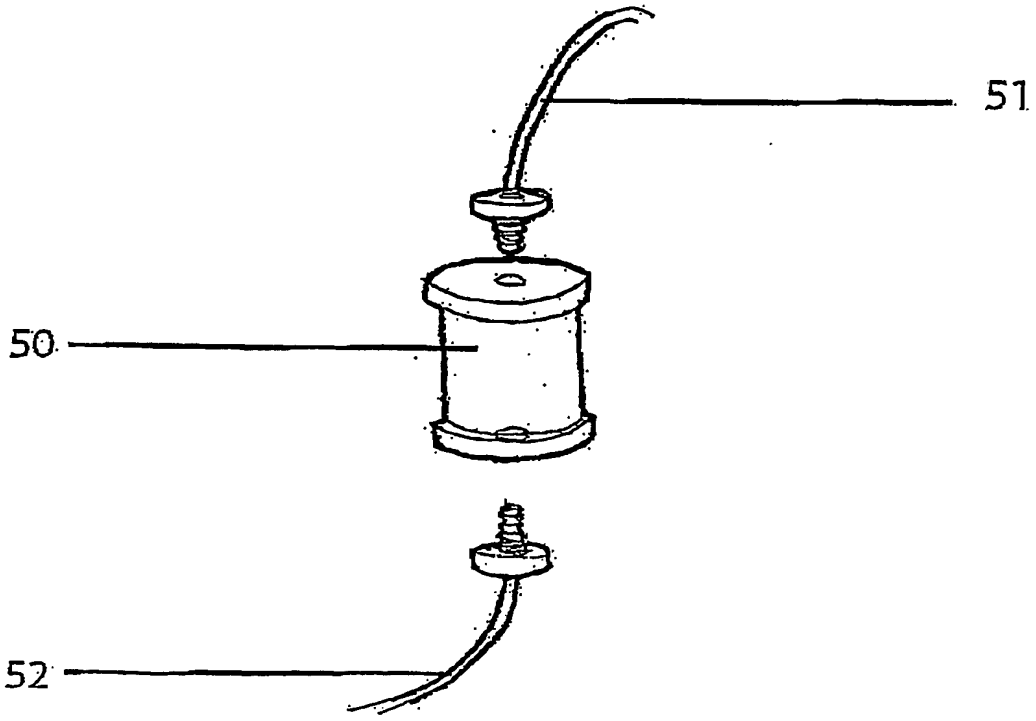


Fig.16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU01/00871

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: C07H 1/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7: AS ABOVE

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CA, WPIDS, Medline searched on STN. Keywords: Glycoprotein?, Mucopolysaccharid?, Mucin(s), Fetuin, Polysaccharid?, Oligosaccharid?, Hydroly?, Alkali?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	The Journal of Biological Chemistry, volume 241, No. 5, issued 25 June 1966, Carlson, Don M. "Oligosaccharides isolated from pig submaxillary mucin", pages 2984-2986	
X	Page 2984 last paragraph	1
	Glycoconjugate Journal, volume 15, no. 10, October 1998, Aeed, Paul A. et al., "Characterization of the O-linked oligosaccharide structures on P-selectin glycoprotein ligand-1 (PSGL-1)", pages 975-985	
X	Abstract	1-4, 6, 8-10, 17

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search	Date of mailing of the international search report
28 September 2001	5 OCTOBER 2001
Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	GAVIN THOMPSON Telephone No : (02) 6283 2240

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Biochemistry, volume 32, No. 2, 1993, Patel, Thakor et al., "Use of Hydrazine to Release in Intact and Unreduced Form both N- and O-Linked Oligosaccharides from Glycoproteins", pages 679-693	
X	Abstract	1
	Journal of Biochemistry, volume 111, 1992, Jikibara, Takayuki et al., "Studies on the Uronic Acid-Containing Glycoproteins of Fusarium sp. M7-1:III. The Primary Structures of the Acidic Polysaccharides of the Glycoproteins", pages 236-243	
X	Abstract	1
	Carbohydrate Research, volume 138, 1985, Neeser, Jean-Richard, "G. L. C. of O-Methyloxime and Alditol Acetate Derivatives of Neutral Sugars, Hexosamines, and Sialic acids: "One Pot" Quantitative Determination of the Carbohydrate Constituents of Glycoproteins and a Study of the Selectivity of Alkaline Borohydride Reductions", pages 189-198	
X	Abstract	1
	The Journal of Biological Chemistry, volume 259, issued 25 October 1984, Rana, Surjit S. et al., "Purification and Structures of Oligosaccharide chains in Swine Trachea and Cowper's Gland Mucin Glycoproteins", pages 12899-12907	
X	Abstract	1
	Canadian Journal of Biochemistry and Cell Biology, volume 62, no. 11, November 1984, Brockhausen, Inka et al., "Mucin synthesis. The action of pig gastric mucosal UDP-GlcNAc:Gal-beta1-3(R_1)GalNAc-R_2(GlcNAc to Gal) beta3-N-acetylglucosaminyltransferase on high molecular weight substrates", pages 1081-1090	
X	Abstract	1
	European Journal of Biochemistry, volume 182, no. 1, 1 June 1989, Capon, Calliope et al., "Structures of O-glycosidically linked oligosaccharides isolated from human meconium glycoproteins", pages 139-152	
X	Abstract	1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU01/00871

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Cancer Research, volume 44, April 1984, Chandrasekaran, E. V. et al, "Structures of the Oligosaccharides Chains of Two Forms of Alpha 1-Acid Glycoprotein Purified from Liver Metastases of Lung, Colon, and Breast Tumours", pages 1557-1567	
X	Abstract Patent Abstracts of Japan, JP 04-053496 A (NATL FOOD RES INST and NISSHIN FLOUR MILLING CO LTD) 21 February 1992	1
X	Abstract Patent Abstracts of Japan, JP 63-007775 A (OJI KOONSUTAAC KK and OJI PAPER CO LTD) 13 January 1988	1
X	Abstract WO 85/04409 A (ENSOGUTZEIT OY) 10 October 1985	1
X	Abstract WO 93/12243 A (SLOVENSKA TECHNICKA UNIVERZITA) A (24 June 1993)	1 to 3
X	Claim 3	1, 2

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU01/00871

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
JP	04-053496	NONE					
JP	63-007775	NONE					
WO	85/04409	FI	841318	FI	69854	NO	84734
		NO	854734	NO	165299	SE	8505100
		SE	466152	US	4681935	CA	1234803
WO	93/12243	CS	9000630	CS	274918	AU	90395/91

END OF ANNEX